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Pestalotiopsis endophytes from leaves of two orchid species collected in Costa Rica

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Abstract – A survey of endophytic *Pestalotiopsis* associated with two orchid species collected in Coco Island in Costa Rica was carried out. Among the fungi isolated from leaves of the two species, genus *Pestalotiopsis* prevailed. We isolated 29 strains of *Pestalotiopsis*, 9 of which from *Camaridium micranthum* M. A. Blanco and 20 from *Ornithidium adendrobium* (Rchb.f.) M.A. Blanco. On the basis of morphological characters, *Pestalotiopsis* isolates were divided into three different groups but their high similarity do not allow unambiguous species identification. The analysis of internal transcribed spacer (ITS) region of ribosomal DNA was then performed to evaluate the taxonomic position of our isolates.

***Pestalotiopsis* / endophytes / orchids / Costa Rica / ITS**

Résumé – Une étude a été faite sur un échantillon de champignons endophytes qui font partie du genre *Pestalotiopsis*. Ces champignons sont associés à deux espèces d'orchidées et ils ont été récoltés sur l'Île de Coco au Costa Rica. Parmi les champignons isolés des feuilles de ces deux espèces d'orchidées prédomine le genre *Pestalotiopsis*. Nous avons isolé au total vingt-neuf (29) souches de *Pestalotiopsis*, dont neuf (9) sur *Camaridium micranthum* M. A. Blanco et vingt (20) sur *Ornithidium adendrobium* (Rchb.f.) M.A. Blanco. Sur la base des caractères morphologiques, les spécimens de *Pestalotiopsis* ont été divisés en trois groupes. Cependant, l'insuffisance des différences morphologiques n'a pas permis de les identifier et les régions ITS de l'ADN ribosomal ont été analysées pour mieux évaluer l'appartenance des échantillons isolés.

***Pestalotiopsis* / endophytes / orchidées / Costa Rica / ITS**

INTRODUCTION

All microorganisms inhabiting the interior of a vegetable, during their life cycle or part of it, may be considered endophytes (De Bary, 1866). Although first described in the XIX Century, endophytic microorganisms were studied in more details only from the 80's onwards. Fungal endophytes exist in living plant parts, they can be latent, mutualist, and saprobes (Fischer & Petrini, 1992; Arnold *et al.*,

2003; Gonthier *et al.*, 2006), they live within their host plants without causing symptoms of disease (Bacon *et al.*, 2000; Wang *et al.*, 2005). They started to be recognized as being of great importance for the hosts, protecting plants against pests, including insects, nematodes and plant pathogenic fungi and bacteria. Some of them have been identified as able to produce compounds of biotechnological value as antibiotics and antitumor drugs. Lots of studies focused on endophytic fungi of roots and aerial parts of coniferous and angiospermous trees, shrubs and herbs from temperate regions. The endophytic mycobiota of tropical ecosystems have been investigated only recently; first reports, related to the isolation of endophytic fungi from tropical host plants, are from Petrini and Dreyfuss (1981) and Dreyfuss and Petrini (1984) with Araceae, Bromeliaceae and Orchidaceae from French Guiana, Brazil and Colombia, sited all in South America. After that, some groups of plants were investigated for a presence of endophytes, mainly palms and fruit-trees. Excepted isolated studies, orchid-fungus relationships is not clear; in fact most orchids are epiphytic in tropical and subtropical forests, but knowledge about their associations with fungi is confusing (Richardson & Currah, 1995). Mycorrhizas seem to be an important component both in epiphytic orchids and in terrestrial and temperate orchids, but published studies on non-mycorrhizal endophytic fungi are rare. Identification of isolates obtained from surface-disinfected fragments of plant species, as leaves, twigs or inner bark, has shown the presence of different endophytes species (Carroll, 1995; Gonthier *et al.*, 2006). It is of great importance to understand the symbiosis mechanisms as the fungal endophytes may play a key role in determining host plant distribution and diversity.

This study is a preliminary step to investigate endophytic *Pestalotiopsis* species, isolated from leaves of two orchid species previously treated as members of *Maxillaria s.l.,* native in Costa Rica. *Pestalotiopsis* is an anamorphic genus of the family *Amphisphaeriaceae* (Jeewon *et al.*, 2003). These species are ubiquitous in distribution, occurring on a wide range of substrata. Many of them are saprobes while others are pathogenic or endophytic on living plant leaves and twigs (Taylor *et al.*, 2001). *Pestalotiopsis* is a complex genus and consists of members difficult to classify at the species level. Species identification in this genus is based on morphology of the conidia, conidiogenesis and teleomorph association but is known only for few species. Recent molecular studies based on rDNA sequences show that the genus contains distinct lineages based on pigmentation of median cells and on morphology of apical appendages (Jeewon *et al.*, 2002) and examined the relation between species names and host association (Jeewon *et al.*, 2004).

In this paper, on the basis of morphological characters, the *Pestalotiopsis* isolates were divided in three different groups, but few diagnostic characters do not allow species determination. Analysis of internal transcribed spacer (ITS) region of ribosomal DNA was carried out to better evaluate a taxonomic position of our isolates.

MATERIALS AND METHODS

Study area, sampling and fungal characterization

Plants of *C. micranthum* and *O. adendrobium* (Blanco *et al.*, 2007) were collected in Coco Island, located in the Pacific Ocean at 532 km from Cabo Blanco (Costa Rica). The geographic coordinates are: 5°31'00" latitude nord, and

87°04'0" longitude west; the insular surface is 23, 85 square kms. The island is a very important National Park because it is considered a natural laboratory for investigations on species evolution. These plants are often an important components of the epiphytic vegetation in Neotropics. A cloud forest covers the entire surface of the island, reaching its highest elevation at 634 m above sea-level. Both orchid species were epiphytes on *Sacoglottis holdridgei* Cuatrec. Plants were identified according to Dressler (2003) and Blanco *et al.*, (2007).

O. adendrobium were collected in two different Coco Island sites: at 15 m above sea level, 5°32'60.08 N, 87°03'41,3" W, and 310 m above sea-level, 5°31'55.3 N, 87°03'47,8" W; *C. micranthum* were collected at 30 m above the sea level, 5°32'33,08 N, 87°03'10.3" W. Leaves samples were collected from respective plants (two leaves for each plants for five plant species) placed in separate polythene bags and processed within 24 hours from collection. Isolation of endophytes from tissues was carried out by surface-sterilizing method (Bayman *et al*, 1996). Leaves were washed under running water to remove dirts, surface sterilized in 6% H_2O_2 for 3 min and rinsed in sterile distilled water. Three segments (5×5 mm) were cut from each leaf, from distal, central and proximal parts of the blade, and cut to obtain half pieces of each segments. Three pieces of each leaf were plated on a potato dextrose agar (PDA, Oxoid) supplemented with 50 ug/1 of streptomycin to limit bacterial growth. Plates were incubated at 22°C for two weeks and checked regularly. Fungal colonies were transferred to PDA and malt extract agar (MEA, Difco). *Pestalotiopsis* species were identified based on the a colony morphology and on sporulating structures (Steyaert, 1949; 1953; Guba, 1961; Nag Rag, 1993). Samples for DNA isolation were lyophilized and stored at –20°C.

DNA isolation, amplification and sequencing

DNA was extracted from about 5-10 mg of lyophilized mycelia following the protocols previously described for mycorrhizal fungi (Paolocci *et al.*, 1999). The ITS1/ITS4 primer pair (White *et al.*, 1990) was used to amplify ITS region. PCR amplification was carried out in a Gene Amp 9700 Thermal Cycler (Applied Biosystems) with the following cycling parameters: an initial denaturation step at 95°C for 3 min, 25 cycles consisting of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, and a final extension for 7 min at 72°C. All PCR amplifications were performed in a 50-µl reaction mixture containing 200 mM of each dNTP, 10 pmol of each primer, 4 mM $MgCl_2$, 10 mM Tris–HCL pH 9.0, 50 mM KCl, and 2.5 units of Taq polymerase (GE healthcare). All PCR experiments included a negative control (no DNA template). Amplification products were evaluated by electrophoresis in an agarose gel in the presence of a size standard (Gene ruler DNA ladder mix, Fermentas). The PCR products were purified using a Jet-Quick spin column (Genomed) and directly sequenced using ITS1 and ITS4 primers and a BigDye terminator sequencing kit (Applied Biosystems) according to the supplier's instructions. Sequencing reactions were run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). ITS sequences were checked for similarity using a basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). ITS sequences showing the highest score and other ITS sequences representative of the different species of *Pestalotiopsis* genus were retrieved from a GenBank and aligned with C11, C2 and C3 ITS sequences using ClustalX software version 1.8 (Thompson *et al.*, 1997). A neighbor-joining tree was obtained using the software MEGA version 4 (Tamura *et al.*, 2007) based on two-parameter distance model of Kimura (1980).

RESULTS

A total of 29 strains of *Pestalotiopsis* were isolated from the studied orchids. In particular, nine strains were isolated from *C. micranthum*, and eight from *O. adendrobium* plants collected in Coco island sites at 310 m above sea level. Twelve additional strains were isolated from *O. adendrobium* plants collected from a different site 15 m above sea level. A first morphological analysis based on conidial characters: sizes, septation, pigmentation and presence or absence of appendages (Guba, 1961; Nag Rag, 1993; Steyeart, 1949; Sutton, 1980), allowed dividing the isolates in three different groups named C11 from *C. micranthum,* C2 and C3 from *O. adendrobium*. However, morphological characters were not sufficiently informative to identify the species. Our strains isolated from *C. micranthum* (C11) present conidia fusiform, straight, rarely curved, 4-septate, hardly constricted at septa, 25-32 µm long, 5,5 µm wide; the 3 median cells equally dark olivaceous, apical and basal cells hyaline; three apical appendages knobbed, hyaline, 15-50 (av. 30) µm (>25 µm long according to Jewoon *et al.*, 2003); basal appendage hyaline, straight, 4-10 µm long. The strains isolated from *O. adendrobium* (C2-C3) present median cells not concolour (versicolours umber or fuliginous), conidia not constricted at septa, over 20 µm long and over 6 µm wide. In order to gain more insight into the classification of the *Pestalotiopsis* species isolated in this study, a strain from each group (C11-1, C2-1 and C3-1) was subject to the analysis of the ITS region. PCR amplification with ITS1/ITS4 primers pair produced an amplicon of about 550 bp and sequence analysis reveal a presence of polymorphisms, confirming that the strains probably belong to 3 different taxa. Similarity search against GenBank database using blast showed that the obtained sequences are similar to ITS sequences of *Pestalotiopsis* spp. In particular, the strain C11-1 showed high sequence similarity with *P. theae* (Sawada) Steyaert (100% sequence identity, E-value 0.0). Blast analysis of C2-1 and C3-1 ITS sequences produced ambiguous results: both strains, in fact, showed high sequence similarity (99-100% of sequence identity, E-value = 0.0) with several *Pestalotiopsis* species. (e.g *P. clavispora* (G.F. Atk.) Steyaert, *P. sydowiana* (Bres.) B. Sutton, *P. leucothoes* (R.P. White) Steyaert, *P. photiniae* (Thüm.) Y.X. Chen). In order to evaluate more in detail taxonomic group or species to which our *Pestalotiopsis* samples can be ascribed, ITS of reference species were retrieved from GenBank and used to perform a phylogenetic analysis. The sequences considered in the phylogenetic analysis were selected on the basis of the following criteria: 1) the blast results 2) the sequences referenced in published works (Jeewon *et al.*, 2002; Wei *et al.*, 2007).

The phylogenetic analysis (Fig. 1) confirms the blast results. The sample C11-1 forms a highly supported cluster (100% of bootstrap replicates) with *P. theae* (99,6 % of sequence identity). The samples C2-1 and C3-1 form a well supported cluster (82% of bootstrap replicates) with species (*P. photiniae, P. clavispora, P. sodowiana, P. leucothoes*) having nearly identical ITS sequences (99,2% of sequence identity).

DISCUSSION

Recent studies based on phylogenetic analysis of the ITS region, indicate that *Pestalotiopsis* species can be grouped in 3 major clades (X, Y, and Z) corresponding to morphology-based classification systems (Jeewon *et al.*, 2002).

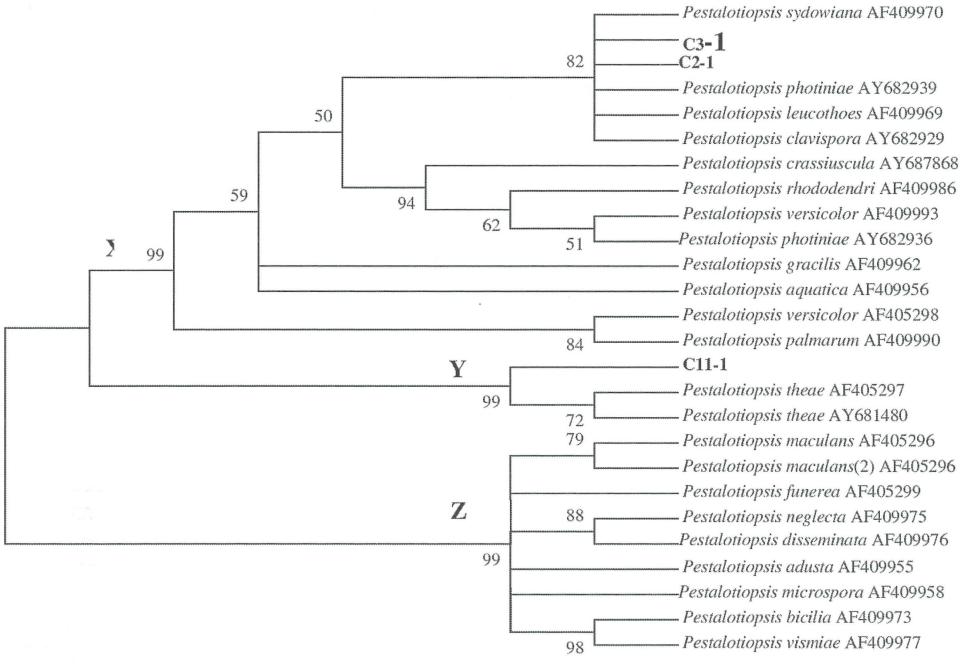

Fig. 1. ITS neighbor-joining treee showing the relationship of C2,C3 and C11 samples with other *Pestalotiopsis* species. Numbers near the branches indicates bootstrap values (percentage over 1000 replicates). X,Y and Z indicate the three major lineages of the *Pestalotiopsis* genus reported by Jewoon *et al.* (2002).

Clade X consists of species with versicolorous median cells; Clade Y and Clade Z species are characterized by brown, concolorous median cells. Other characters for species identification are the apical appendage morphology and length and spore length. From this study a very low variability of the ITS region from *Pestalotiopsis* species emerges, and the analysis of this genomic region does not allow a clear species attribution of the different isolates. As a matter of fact, our analysis of the ITS region, comparing our samples with species analysed in previous studies, allowed the identification of sample C11-1 only. This sample shows high similarity with *P. theae* sequences present in GenBank and this species forms a distinctive clade: the clade Y described by Jeewon *et al.*, (2002).

The isolates C2-1 and C3-1 are both phylogenetically related to species of clade X described by Jeewon *et al.*, (2002). However many species in this clade (e.g. *P. photiniae*, *P. clavispora*, *P. sodowiana*, *P. leucothoes)* have almost identical ITS sequences. *Pestalotiopsis* species are remarkably difficult to identify as many of them have characters largely overlapping. The situation is furthermore complicated as many new species were described because isolated from different hosts. However, recent studies based on the analysis of ITS region of rDNA demonstrate that *Pestalotiopsis* strains do not all have high host specificity, as also suggested for *Colletotrichum* endophytes on orchids (Yang *et al.*, 2011).

Thus, naming species on the basis of host from which these species were isolated does not represent a valid criterion, and it is more than likely that the high number of species reported in the literature is an overestimate of the real number of biological species (Jewoon *et al.*, 2004).

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